

Biogeography and ecology of geographically distant populations of sibling *Cryptocephalus* leaf beetles

D. Kubisz, G. Magoga, M. A. Mazur, M. Montagna, R. Ścibior, P. Tykarski & Ł. Kajtoch

To cite this article: D. Kubisz, G. Magoga, M. A. Mazur, M. Montagna, R. Ścibior, P. Tykarski & Ł. Kajtoch (2020) Biogeography and ecology of geographically distant populations of sibling *Cryptocephalus* leaf beetles, *The European Zoological Journal*, 87:1, 223-234

To link to this article: <https://doi.org/10.1080/24750263.2020.1752832>



© 2020 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group.



[View supplementary material](#)



Published online: 06 May 2020.



[Submit your article to this journal](#)



[View related articles](#)



[View Crossmark data](#)



Biogeography and ecology of geographically distant populations of sibling *Cryptocephalus* leaf beetles

D. KUBISZ ¹, G. MAGOGA ², M. A. MAZUR ³, M. MONTAGNA ², R. ŚCIBIOR ^{4*},
P. TYKARSKI ⁵, & Ł. KAJTOCH ¹

¹Institute of Systematics and Evolution of Animals Polish Academy of Sciences, Krakow, Poland, ²Dipartimento di Scienze Agrarie e Ambientali, Università degli Studi di Milano, Milan, Italy, ³Institute of Biology, University of Opole, Opole, Poland, ⁴Department of Zoology and Animal Ecology, University of Life Sciences in Lublin, Lublin, Poland, and ⁵Department of Ecology, Faculty of Biology, Biological and Chemical Research Centre, University of Warsaw, Warsaw, Poland

(Received 18 November 2019; accepted 11 March 2020)

Abstract

Different populations of two closely related species, *Cryptocephalus flavipes* and *C. bameuli*, from western (Alps, Apennines and Pyrenees) and central Europe (Poland, Ukraine and Pannonia) were analysed. On the basis of DNA sequences from two genes, *cox1* and *ef1-α*, distinctiveness of both species was confirmed. Nevertheless, possible hybrids were identified in Carpathian mountains. We found a significant genetic differentiation among populations of *C. flavipes* and *C. bameuli* from distant regions but a high genetic similarity between populations of *C. bameuli* from north and south of the Carpathians. Demographic estimates suggest a past population expansion in the case of *C. bameuli* and a recent one for *C. flavipes*, possibly occurred during Pleistocene and Holocene, respectively. Distribution modelling showed that *C. flavipes* is typically present in the mountain systems, whereas *C. bameuli* is associated with hilly areas of central and eastern Europe. Based on the present data, Last Glacial Maximum refugia of both species were located in the Alpine region and Black Sea coasts, but on different elevations. The characterization of the insect diet, through a DNA metabarcoding approach targeting the *trnL* plant intron, demonstrated a significant differentiation of food preferences between the two species, as well as between geographic populations within the species.

Keywords: Chrysomelidae, Coleoptera, hybridization, molecular ecology, phylogeography, niche modelling

Introduction

Cryptocephalus Müller (Chrysomelidae) is one of the most species-rich genera in the order of Coleoptera, and because of that, it represents an excellent model for studying ecological and evolutionary processes, in particular speciation. Recent studies focused on leaf beetles have revealed interesting evolutionary features of *Cryptocephalus*, as the presence of highly divergent lineages (i.e. *Cryptocephalus coryli* (L.), *C. decemmaculatus* (L.), *C. nitidulus* (F.) and *C. barii* Burl.) (Piper & Compton 2003; Brunetti et al. 2019); cases of hybridization and mtDNA paraphyly in the only two species complexes studied so far (i.e. *C. sericeus* and *C. flavipes* species complexes; Gómez-Zurita et al. 2012; Montagna et al.

2016a). The latter finding was explained by the authors as the result of recent cladogenetic events and by episodes of hybridization in areas of secondary contact among the closely related species.

The *C. flavipes* complex represents an interesting case study for population genetics and ecology (e.g. the host plants preferences) of sibling species (Lopatin & Nesterova 2002). *C. flavipes* complex includes eight taxa with highly similar external morphologies; the species have medium size (2.5–5.0 mm) and are distinguishable by slight differences in the shape of the spermatheca and by patterns of yellow stripes on the pronotum and elytra margins (Duhaldeborde 1999; Warchałowski 2010). Recently, the shape of the metaepisternum

*Correspondence: R. Ścibior, Department of Zoology and Animal Ecology, University of Life Sciences in Lublin, Lublin, Poland.
Email: radoslaw.scibior@up.lublin.pl

has been demonstrated to be a highly effective character in differentiating among the species of the complex (Montagna et al. 2016a). At present, the following species are listed within the complex: *C. signatifrons* Suffr. (distributed in Europe), *C. turcicus* Suffr. (present in the Mediterranean Basin and the Near East), *C. peyroni* Mars. (Near East), *C. bameuli* Duha. (Europe to West Asia), *C. flavipes* F. (Europe to West Asia), and *C. alborzensis* Rapi. (Near East). Recently, integrating molecular and morphological data, *C. quadripustulatus* Gyll. (distributed in Europe) was assigned to the *C. flavipes* group and the existence of a new taxon from Turkey was demonstrated adopting an integrated taxonomic approach (Montagna et al. 2016a), which it was formally described as *C. cilicus* by Duhaldorde (2018). *Cryptocephalus flavipes* and *C. bameuli* inhabit similar environments such as the xeric grasslands and the scrublands. However, the localization of known populations of the two species suggests that *C. flavipes* is more associated with dry scrublands and woodland edges of mountain areas, whereas *C. bameuli* inhabits mainly steppes and xeric grasslands. Because of their similar phenotypes and ecology, in association with the hybridisation hypothesis (Montagna et al. 2016a), these species can be considered excellent subjects to investigate evolutionary processes that led to their separation, as the past-present population diversity and demography, host plants adaptation and microbiota associations, as postulated in the case of the *C. marginellus* species complex (Montagna et al. 2015b).

Using molecular data and species distribution modelling analyses, three main goals have been addressed in the present study: i) estimate the genetic diversity and distinctiveness among geographically distant European populations of *C. flavipes* – *C. bameuli*; ii) infer their demographic history during the Quaternary climatic fluctuations, in order to evaluate the responses of the two species and their potential glacial refugia; iii) identify the range of host plants exploited by the species, through a DNA metabarcoding approach.

Material and methods

Sampling

We used the collection of specimens of *C. flavipes* and *C. bameuli* from the majority of their ranges in Europe, previously used in the work of Montagna et al. (2016a) (Supplementary Table I, Supplementary File I). See for details about the

preservation and identification of specimens in Montagna et al. (2016a).

In order to perform the subsequent analyses, the collected beetles were clustered into the following four major groups according to the collection localities: i) *C. flavipes* from Alps and Apennines; ii) *C. bameuli* from the Alps (population from the Pyrenees was excluded due to low sampling and genetic monomorphism of individuals), iii) *C. bameuli* from the north of the Carpathians (Poland and Ukraine); and iv) *C. bameuli* from the south of the Carpathians – Pannonia. Such geographic grouping follow current knowledge on the phylogeography of beetles related with steppes and other xeric habitats of Europe (see Kajtoch et al. 2016) and is justified by phylogenetic trees presented in the preceding study (Montagna et al. 2016a).

In the niche modelling analyses we used geographic coordinates of the specimens collected during 2008–2014 field trips; in addition, the geographic coordinates of the specimens from Central, Eastern and South-Eastern Europe preserved in museum collections (Nature Museum of Institute of Systematics and Evolution of Animals PAS, Museum and Institute of Zoology PAS, Upper Silesian Museum in Bytom) were included in the analyses. The analyzed dataset includes specimens collected in 25 localities for *C. flavipes*, and in 32 localities for *C. bameuli* (Supplementary Table II).

DNA extraction and PCR amplification

DNA isolation and sequencing of two genes (mitochondrial Cytochrome Oxidase I; *cox1* and one nuclear Elongation Factor 1 alpha; *ef1- α*) were described in the earlier work of Montagna et al. (2016a).

In order to characterize the host plants of the two species, DNA isolates from the whole organism were used to amplify *trnL(UAA)* plant intron using A49325/B49863 primers (Taberlet et al. 1991), successfully used for leaf beetles (e.g. Montagna et al. 2013; De la Cadena et al. 2017). The choice of this marker was also dictated by previous development of barcode database for xeric plants of Central Europe (Heise et al. 2015), which was crucial for plant taxa assignment (see further chapter for details). PCR amplicons were obtained from the four geographical areas previously defined; the PCR amplicons were then mixed according to the region of collection and normalized, tagged and run on Illumina MiSeq 2 × 150 bp read lengths (using a MiSeq Reagent Kit v2).

All the *cox1* and *ef1- α* sequences are available in GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) under accession numbers: KJ765872–KJ765907 and KM243298 for *cox1*; KJ765908–KJ765932 and KM243299 for *ef1- α* . Plant barcodes obtained with the use of high throughput sequencing are submitted to Sequence Read Archive (<https://www.ncbi.nlm.nih.gov/sra>) under accession numbers: SAMN13855172–175, SRR10899136–139.

Population genetic analysis

Standard genetic indices such as haplotype diversity (h), nucleotide diversity (π), and the number of private haplotypes (N_p) for populations were computed with DnaSP v.5 (Librado & Rozas 2009). Ambiguous nucleotides in *ef1- α* gene sequences were resolved through the use of the Unphased/Genotype Data option of DnaSP v.5 (Librado & Rozas 2009). The HAPAR (Wang & Xu 2003) algorithm was used to infer the haplotype sequences. These haplotypes were also inspected by eye and compared to other *ef1- α* sequences. Haplotype networks of *cox1* and *ef1- α* sequences were constructed using the Median-Joining network method (Bandelt et al. 1999) in the Network 4.6.1.0 software (<http://www.fluxus-engineering.com/>).

Demographic and migration analyses

A mismatch distribution (MD) (Rogers & Harpending 1992) on *cox1* sequences was calculated in ARLEQUIN 3.5 (Excoffier & Lischer 2010) in order to examine demographic history, and specifically to test for historical (temporal) expansions of both species populations (in the case of *C. bameuli*, separately for each regional group of populations, as previously defined). The probable expansion time was estimated by the upper and lower limits of the τ parameter, adopting the Coleopteran evolutionary mean rate of 0.0177 (SD = 0.0019) for the mitochondrial *cox1* (Papadopoulou et al. 2010) and using one generation per year.

Niche modelling

For environmental niche modelling (ENM) was used the MaxEnt algorithm (MaxEnt Model v3.3.3 k available at www.cs.princeton.edu/~schapire/maxent; Phillips et al. 2006), with the following settings: number of iterations = 500, convergence threshold = 10^{-5} , regularization multiplier = 1; maximum number of background points = 10^3 . The run was repeated 10 times, with cross-validation as

a method for evaluation, and adding samples to the background to encompass all combinations of environmental data present in the data points. We used a mask in order to exclude certain areas from background points available for sampling that are beyond a known range of a species (Merow et al. 2013), confining the extent to 12° W, 66° N, 60° E, 30° N (excluding Iceland and areas beyond the Polar Circle). Area under the curve (AUC) of the receiver operating characteristic (ROC) plot was used as a measure of accuracy of the models produced.

For contemporary distribution models, we used 19 bioclimatic variables from the WorldClim data set (Hijmans et al. 2005), generated at 2.5-arc-minute resolution. The model was projected onto LGM data, reconstructed and made available by University of Miami – RSMAS (CCSM4), with the same variables and resolution. All climatic layers were downloaded from the website www.worldclim.org. In order to determine the influence of the variables in the model were calculated the MaxEnt's heuristic estimates: the percentage contribution and the permutation importance, as well as the jackknife test performed for each of the variables separately (Phillips & Dudík 2008).

Host plant identification

The reads of *trnL* intron were identified by megablast analyses (E value cut-off = $1e-20$; Altschul et al. 1990) and by comparison with the accurate *trnL* dataset obtained for dry grassland plants from central and eastern Europe (Heise et al. 2015). The methods used for this purpose (including thresholds of considered values) follow procedures adopted in Kajtoch et al. (2015). A read was considered to have a unique hit only if a single hit was reported or when the bitscore of the second-best hit was not greater than 0.95 x the bitscore of the best hit. When this condition was not met, then all hits (species) with the bitscores >0.95 x the bitscore of the best hit were considered as matching the read equally well. The obtained *trnL* illumina reads were identified at genus and species level (when possible) and then arranged in the operational taxonomic units (OTU) table in order to perform the comparative analyses. Diversity indices calculation and the following analyses (exceptions are specified) were performed using the software R package (R Project 3.0.2; <http://cran.r-project.org/>) package *vegan* (Dixon 2003). In order to test dissimilarities between the host plant communities associated with the four groups of specimens (i.e. *C. bameuli* from (i) the Alps, (ii) area north of the Carpathians, (iii) area south of the Carpathians; and (iv). *C. flavipes* from the Alps–Apennines), the dissimilarity matrices, obtained as

previously described in Montagna et al. (2015a, 2016b), were subjected to a nonparametric one-way analysis of similarity (ANOSIM; Clarke 1993) as in a previous work (Montagna et al. 2015b). To investigate the OTUs shared among the four analyzed groups, an analysis of commonality was performed and visualized through Venn diagrams using the *g-plots* package in R.

Results

Population genetics and demographic history

The mitochondrial genetic diversity of the populations of *C. flavipes* and *C. bameuli* was found to be moderate or high, generally higher in the latter species (Table I); this observation could be the result of the unequal sample size. However, even in the case of an identical sample size ($N = 9$ for both), as for the Alpine populations, *C. bameuli* resulted to be more diverse than *C. flavipes* (mean Hdiv = 0.83–0.90 and 0.22, respectively). The most diverse populations of *C. bameuli* were localized in the Alps (mean Hdiv = 0.83–0.90), and in the north of the Carpathians (southern Poland) (mean Hdiv = 0.90). The populations from Pannonia (south of the Carpathians) (mean Hdiv = 0.60–0.70) and Ukraine (northeast of the Carpathians) (mean Hdiv = 0.53)

were less diverse. No genetic diversity was found among the specimens from Pyrenees. On the other hand, with respect to the nuclear *ef1- α* gene, both species were found moderately diverse (mean Hdiv = 0.40–0.70), but some populations resulted monomorphic. In the single Carpathian locality was determined hybrid population of *Cryptocephalus* beetles. The inclusion of that population increases the values of genetic indices of all populations of *C. flavipes*, erroneously suggesting that this species has greater genetic diversity than *C. bameuli*. The Carpathian population was included in *C. flavipes* as its individuals have morphology resembling *C. flavipes*, despite their hybrid origin.

Haplotype networks constructed using *cox1* and *ef1- α* haplotypes supported the distinctiveness of both the two species (Figure 1). In addition, the networks showed a higher diversity for *C. bameuli* populations, at least with respect to the *cox1* marker, confirming previous results. There were interesting findings concerning the position of three specimens from the Carpathians that were morphologically identified as *C. flavipes* (identifiers F-KP1, F-KP2 and F-KP3). In the *cox1* network, a single individual from the Carpathians (F-KP2) represented a distinct isolated lineage compared to other *C. flavipes* haplotypes from Italian Alps, whereas the haplotypes of F-KP1 and F-KP3 clustered

Table I. Basic genetic diversity indices of *cox-I* and *ef1- α* markers calculated for *C. flavipes* and *C. bameuli*. n – sample size, V – variable sites, Hnum – haplotype number, Hdiv – haplotype diversity with SE, π – nucleotide diversity with SE, S – number of segregating sites, Hpriv – number of private haplotypes.

marker	<i>cox-I</i>						<i>ef1-α</i>						
	n	V	Hnum	Hdiv	π	S	Hpriv	V	Hnum	Hdiv	π	S	Hpriv
<i>C. flavipes</i>													
all *	23	101	10	0.81 ± 0.07	0.0286 ± 0.0103	91	-	4	4	0.46 ± 0.115	0.0014 ± 0.0005	2	-
all Italian	14	3	4	0.65 ± 0.12	0.0012 ± 0.0008	2	-	1	2	0.40 ± 0.11	0.0009 ± 0.0007	1	-
Alps	9	1	2	0.22 ± 0.17	0.0003 ± 0.0005	0	2	1	2	0.51 ± 0.10	0.0011 ± 0.0007	1	1
Apennines	5	1	2	0.60 ± 0.18	0.0008 ± 0.0007	1	2	0	1	0	0	0	0
Carpathians *	3	77	3	1.00 ± 0.27	0.0707 ± 0.0425	0	3	7	3	0.70 ± 0.22	0.0073 ± 0.0027	3	2
<i>C. bameuli</i>													
all	71	58	24	0.90 ± 0.02	0.0111 ± 0.0046	49	-	3	5	0.64 ± 0.03	0.0017 ± 0.0008	3	-
Alps-Italy	5	11	4	0.90 ± 0.16	0.0082 ± 0.0041	8	4	0	1	0	0	0	0
Alps-France	4	3	3	0.83 ± 0.22	0.0021 ± 0.0000	0	3	1	2	0.53 ± 0.17	0.0012 ± 0.0009	1	2
Pyrenees-France	5	0	1	0	0	0	1	0	1	0	0	0	1
Hungary	10	2	3	0.60 ± 0.13	0.0009 ± 0.0007	1	1	0	1	0	0	0	0
Czech+Slovakia	14	8	5	0.66 ± 0.12	0.0036 ± 0.0017	5	3	0	1	0	0	0	0
+Austria													
Poland S	12	11	7	0.91 ± 0.06	0.0054 ± 0.0024	9	6	1	2	0.49 ± 0.11	0.0007 ± 0.0007	1	0
Poland N	9	0	1	0	0	0	1	0	1	0	0	0	0
Ukraine	12	5	3	0.53 ± 0.14	0.0028 ± 0.0013	4	2	0	1	0	0	0	0

* Note: In the single Carpathian locality was determined hybrid population of *Cryptocephalus* beetles. Including of that population increase the values of genetic indices of all populations of *C. flavipes*, what erroneously suggest that this species has greater genetic diversity than *C. bameuli*. The Carpathian population was included in *C. flavipes* as individuals found there are morphologically assigned to *C. flavipes*, despite their hybrid origin.

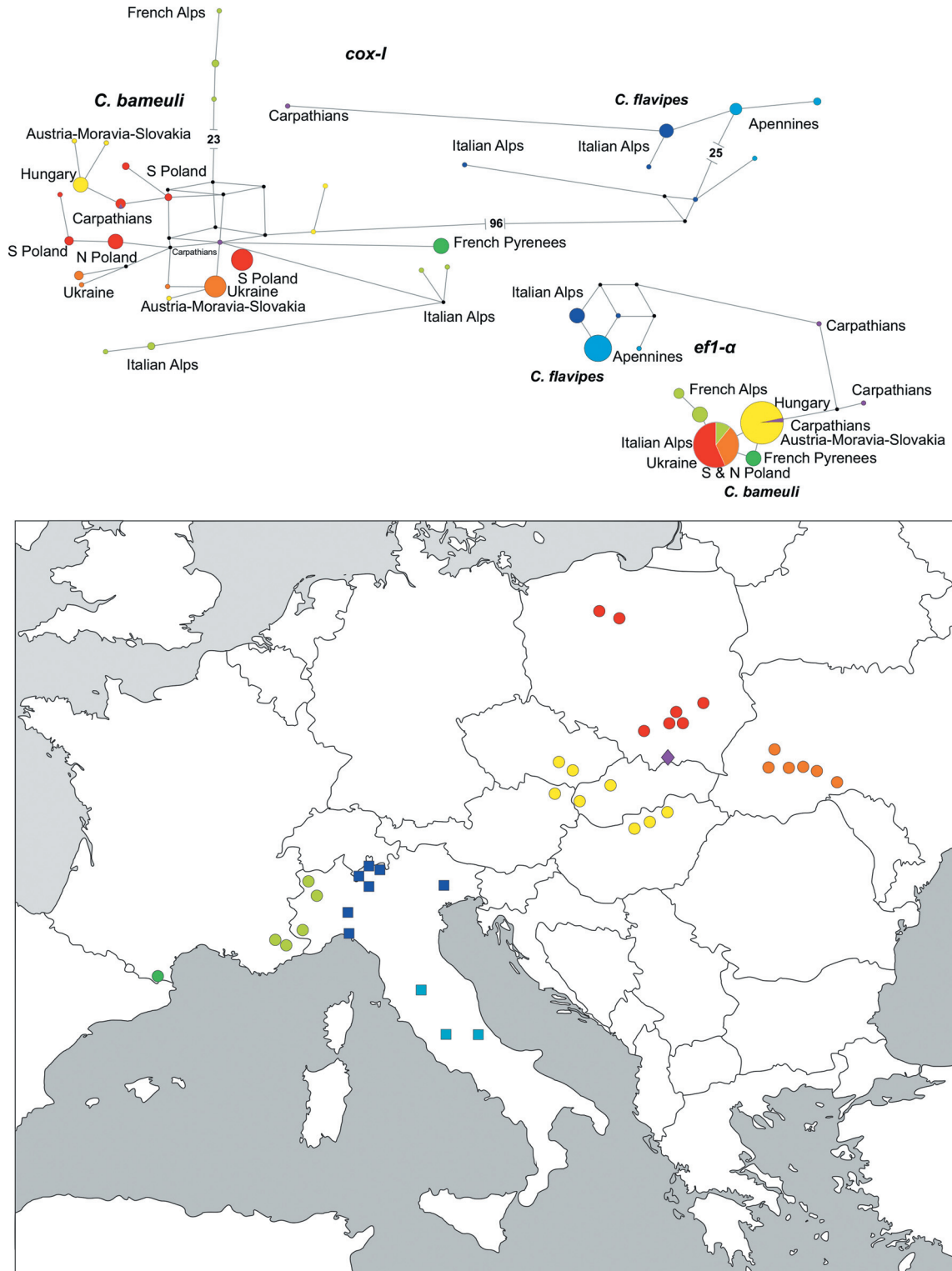


Figure 1. Map with localization of sampling sites and *cox-I* and *ef1- α* median joining networks constructed for haplotypes of *Cryptocephalus flavipes* (squares on the map) and *C. bameuli* (circles) species and probable hybrids (diamond). Colours correspond to sampling sites presented on the map (sites from geographic regions are marked with the same colour). Sizes of circles on networks correspond to the number of specimens. Black dots – missing haplotypes. Numbers – mutations on the longest lines connecting distant haplogroups.

within the *C. bameuli* clade. In the network based on the *ef1-a* gene, the two haplotypes of Carpathian specimens (F-KP1 and F-KP2) seemed to be intermediate between the clades of *C. flavipes* and *C. bameuli*. The third individual from the Carpathians (F-KP3) possessed the most common haplotype of *C. bameuli* in Pannonia (Figure 1).

Demography

Mismatch distribution analysis suggested expansion events of the examined populations in different periods. The population of *C. bameuli* from Alps-Pyrenees suffered the most ancient event of expansion 129 Kya (156-75 Kya), while central European populations could have expanded in similar ways, but in more recent times (~22.4 Kya and ~24.0 Kya, respectively). On the other hand, *C. flavipes* from Alps and Apennines expanded very recently, approximately 3.5 Kya (Supplementary Table III, Supplementary Figure 1).

Niche modelling

The niche models of both species are rather strongly supported, according to AUC values: 0.939 ± 0.043 for *C. bameuli* and 0.970 ± 0.036 for *C. flavipes* (Supplementary Table IV). Regularized gain was 1.825 for *C. bameuli* and 2.396 for *C. flavipes*.

Regarding *C. bameuli*, the most contributing variables were isothermality (contribution 36.3%, permutation importance 6.3) and precipitation of driest month (25.5%, 6), precipitation of coldest quarter (5.9%, 17.7), for *C. flavipes* the most important variables resulted to be isothermality (19.1%, 4.1), precipitation of wettest quarter (18%, 45.4), precipitation of warmest quarter (12.5%, 0) and of the driest month (12.4%, 6.6).

When tested in isolation (jackknife test), the variables of the highest gain were isothermality (0.972) for *C. bameuli* and precipitation of the wettest quarter (1.186) for *C. flavipes*. During the LGM, the extension of the *C. bameuli* predicted niche is broader, then that inferred for *C. flavipes* (Figure 2a,b). The split into four classes (limited with 0.25, 0.50, 0.75, and 1)

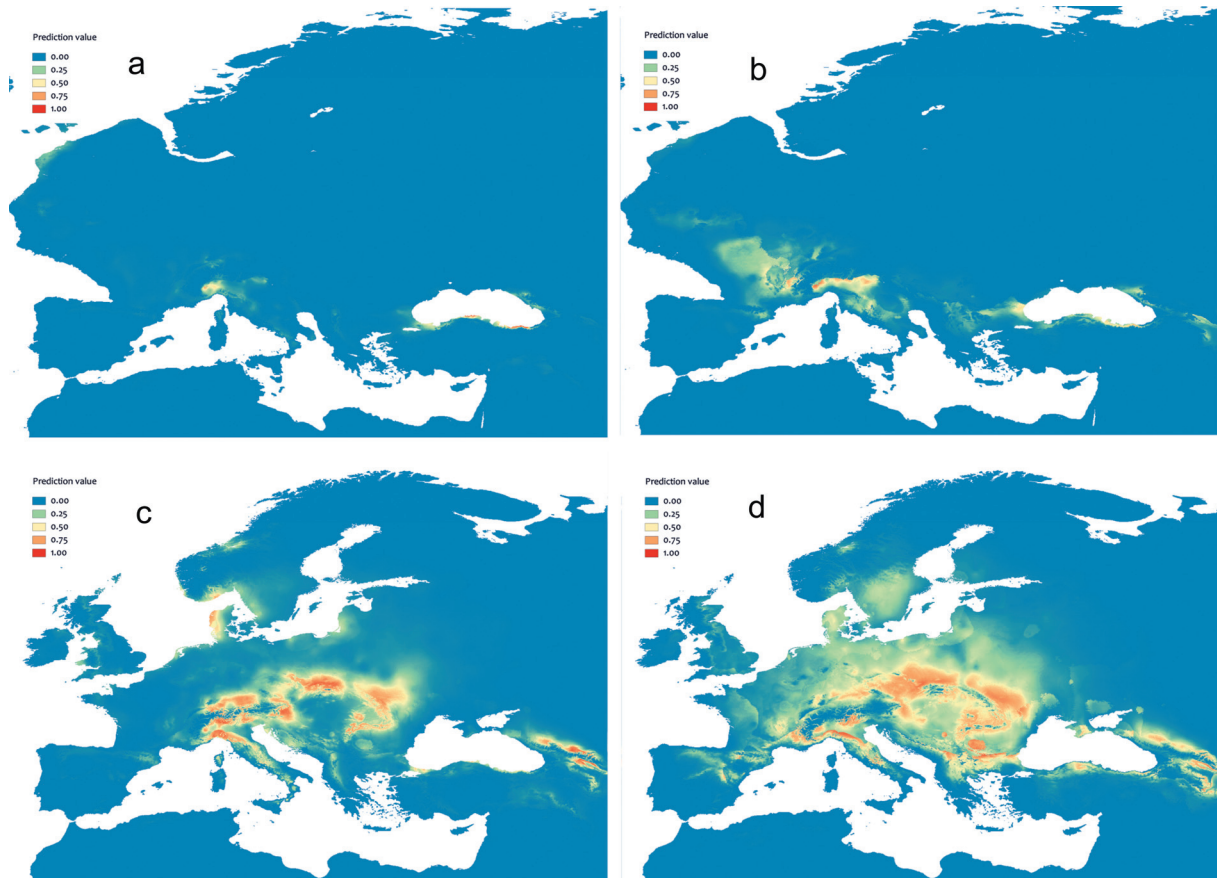


Figure 2. Environmental niche modelling with the use of MaxEnt algorithm and WorldClim data set for contemporary (a&b) and Last Glacial Maximum c&d) modelling for *Cryptocephalus flavipes* (a&c) and *C. bameuli* (b&d) populations.

of the predicted suitability ranges showed that under the current climatic conditions, *C. bameuli* areas of suitability (higher than 0.25 and higher than 0.5) are, respectively, about 2.3 and 1.8 times bigger than those of *C. flavipes* (Supplementary Table V). The inferred niche models for LGM showed similar proportions of the suitability areas. At Figure 2(c,d) are shown on maps the models of the current distribution of the species.

In terms of the current geographic distribution, *C. flavipes* resulted more confined to the mountain environments (Figure 2c) while *C. bameuli* found to be widespread in various elevations (Figure 2d). The latter finds suitable living conditions also at lower elevations from western Europe to the Middle East and has its northern limit in the south of Scandinavian Peninsula (Figure 2d); the mountains of the northern Balkan Peninsula appeared to be suitable for *C. bameuli* but not for *C. flavipes*. Under LGM conditions, the inferred niches were less extended; suitable areas for *C. bameuli* were Jura Mountains, Po Valley, and the coast of the Black Sea, whereas *C. flavipes* was restricted to the Alpine region and to northern Anatolia (Figure 2a,b).

Host plants

The analyses performed on the *trnL* Illumina reads confirmed the polyphagy of *C. flavipes* and *C. bameuli*. Indeed, they revealed 56 plant taxa (SD \pm 11.1) on average associated with the three analyzed groups of *C. bameuli* (i.e. specimens collected from the Alps, north and south of Carpathians), while, a total of 58 plant taxa present in the diet of *C. flavipes* from the Alps-Apennines (Figure 3). Interestingly, no significant differences were present in the diet composition of the three analysed groups of *C. bameuli* (ANOSIM analysis), while some differences were recovered when the diet composition of *C. flavipes* from the Alps-Apennines was included in the analysis (p-value = 0.042).

Regarding the taxonomical aspect of diet composition, several plant genera and species, mainly belonging to Rosaceae, Fabaceae, and Betulaceae, were identified in the diet of the regional populations of both species (Figure 3a). At the family level, no differences were observed among the groups of *C. bameuli*, whereas in the diet of *C. flavipes* were not present members of Asparagaceae, which instead resulted abundant in all groups of *C. bameuli*. Plant taxa shared among the three groups of *C. bameuli* and between *C. flavipes* and *C. bameuli* from the Alps were investigated using

Venn diagrams (Figure 3b). As expected, the three groups of *C. bameuli* shared a core diet, encompassing 35 taxa of plants (e.g. *Onobrychis vicifolia*, *Prunus spinosa*, *Rosa canina*). Moreover, unique plant taxa were recovered in all of them, one in the populations from the Alps-Apennines, two and ten, respectively, in those inhabiting the areas in north and in south of the Carpathians. In detail, the analysed specimens of *C. bameuli* from Alps-Apennines mostly fed on plants of the genus *Lotus* and *Onobrychis* but also *Rosa* and *Corylus*; however, populations from both north and south of the Carpathians fed primarily on *Crataegus*, *Prunus*, *Rosa*, and on some herbs belonging to Rosaceae as well as on *Anthericum*, *Asparagus*, *Genista* and *Onobrychis*. The comparison between *C. flavipes* and *C. bameuli* inhabiting the same geographical area (i.e. Alps-Apennines) led to similar results, with 34 plant taxa (including, e.g. *Corylus*, *Crataegus*, *Lotus* and *Onobrychis*) shared among them out of a total of 68 (58 associated with *C. flavipes* and 44 with *C. bameuli*).

Discussion

Different evolutionary histories

Population genetic analyses, niche modelling and the estimation of the demographic changes in the populations of *C. flavipes* and *C. bameuli* show that these two sibling species experienced rather different demographic histories and that these differences are not only species-specific but also geographically related. Interesting is that for both species similar sets of bioclimatic variables found to be crucial, that isothermality and precipitation. The same bioclimatic variables were also mostly responsible in niche modelling of other *Cryptocephalus* (e.g. *C. barii*, Brunetti et al. 2019), suggesting that the species of this genus respond to a similar set of climatic factors, however not necessarily in the same direction. So far, it was assumed that *C. flavipes* was widespread across the temperate zone of Europe and the Near East (Löbl & Smetana 2010); however, in some areas, its presence should be verified due to possible misidentifications of the two species. Our study does not allow for a definitive estimation of the *C. flavipes* range, but niche modelling suggests that it is restricted to the mountain systems across Europe; contrary to the much wider range reported by the Catalogue of Palaearctic Coleoptera (Löbl & Smetana 2010), our analyses showed that the species should be absent in the Balkans and in Anatolia. It is noteworthy that we found *C. flavipes* in a single locality

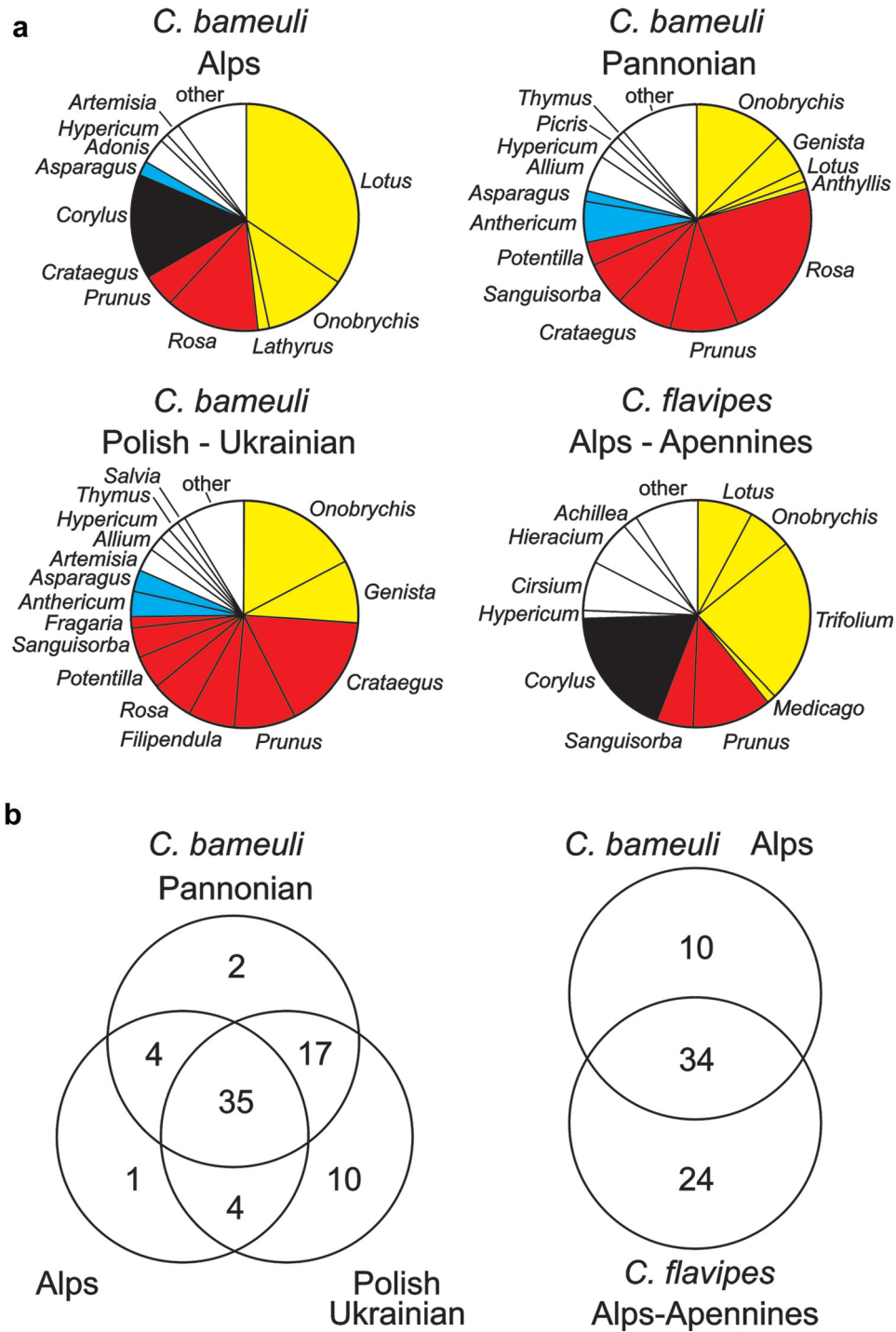


Figure 3. Relative share of host plant of three studied groups of *C. bameuli* (from Alps, Pannonian region and Poland-Ukraine) and one of *Cryptocephalus flavipes* (from Alps-Apennines). (a). Pie charts representing the taxonomic assignment at genus level of plant *trnL* gene sequences; yellow (light grey): Fabaceae; red (dark grey): Rosaceae; blue (medium grey): Asparagaceae; black: Corylaceae, and white: other plant families. (b). Venn diagram showing shared plant OTUs between the compared groups.

in the Carpathians, despite the extensive collecting campaigns performed from 2008 to 2014 (~30 collecting campaigns). Even if *Cryptocephalus* species seem to persist at very low population densities

(Piper 2002), our findings suggest that most of the *C. flavipes* specimens collected across central and eastern Europe and deposited in museum collections should be revisited. On the other hand,

C. flavipes is widespread and abundant in the Alps and the Apennines. Demographic data estimated for the analysed Italian populations of this species suggest a possible expansion just in the middle of the Holocene period. The most probable explanation for the obtained pattern is that *C. flavipes* survived to the less favourable conditions of Pleistocene glaciations in refugia located approximately in the same areas where it is present now; this result is consistent with the predicted species distribution during LGM. The finding of phylogenetically divergent lineages in the Carpathians suggests the presence of a LGM refugium within the Carpathian Basin, which has been recently confirmed as an important extra-Mediterranean refugium (see Stewart & Lister 2001; Schmitt & Varga 2012). Niche modelling failed to locate this refugium, but it could be the consequence of a bias induced by the relatively low number of sampled sites in Central Europe. Conversely, genetic data and niche modelling analyses on *C. bameuli* suggested that this species experienced quite the opposite historical events. The fact that the subalpine populations of this species proved to be the most genetically diverse suggests the role of refugium of the Alps, especially of their western slope; this statement is also supported by the niche modelling. A further refugium could have existed in the Pyrenees; however, the population from this area proved to be genetically monomorphic. The observed genetic variability might be an effect of the low sample size ($N = 5$) or, most likely, of the high level of inbreeding of the population in which the specimens were collected. The western population of *C. bameuli* could have experienced an expansion between 170 Kya and 36 Kya. On the other hand, *C. bameuli* populations could have expanded to central and eastern Europe during the LGM or soon after it; this is consistent with the predicted species distribution during LGM (restricted to areas around the Alps and the Black Sea) and with present distribution (high probability of occurrence across hilly areas of Central, South-Eastern and Eastern Europe). The differences in the time of *C. bameuli* population expansions are not easily explained, but they might be linked to the different environmental and climatic conditions present in the subalpine areas and around the Carpathians. The glaciated areas during LGM were more extensive in central and eastern than in western (especially southwestern) Europe. The current south Alpine piedmont was generally ice-free, whereas an ice sheet covered most of the areas north of the Carpathians (Ehlers & Gibbard 2004). This assertion, however, does not explain the similar and

relatively late expansion of Pannonian populations, but the observed patterns for populations north and south of the Carpathians suggest that *C. bameuli* could have expanded from some eastern refugia (e.g. Black Sea coast, as it was predicted in niche modelling) and colonized both sides of the Carpathians in the same time. The estimates of the time of expansion for the populations of *C. bameuli* strongly suggest that this species is much more cold-tolerant than *C. flavipes*. Climate warming could lead to the further expansion of *C. flavipes* and to the retreat of *C. bameuli*, at least from the southernmost localities. The latter scenario was also recently predicted for cold-adapted mountain *C. barii* in the Alps (Brunetti et al. 2019).

The different stories experienced by the two species suggest that despite their similar morphologies, they are probably adapted to different climatic and environmental conditions (including some differences in food preferences, as seen below). We hypothesize that *C. flavipes* might be associated with mountain Mediterranean xeric habitats on limestone, whereas *C. bameuli* is probably more related to continental steppes. Differently to the majority of steppic beetles (see Kajtoch et al. 2016 for summary), the populations of *C. bameuli* from the inner and outer side of the Carpathians express a high level of genetic similarity. The haplotypes in common among *C. bameuli* populations living north and south of the Carpathians could be a consequence of (i) a recent expansion of this species in central and eastern Europe, as supported by demographic analyses and niche modelling and (ii) current high migration rates. The limited dispersal abilities of *Cryptocephalus* beetles (e.g. as demonstrated for *Cryptocephalus decemmaculatus* and *Cryptocephalus nitidulus*; Piper & Compton 2010, 2013) support the first scenario. On the other hand, shared haplotypes were detected only in adjacent areas on both sides of the Carpathians (Moravia and southern Poland), suggesting a limited bidirectional dispersal of individuals in current times. However, both explanations are not exclusive since the recent species expansion across the Carpathians could have been continued via, e.g., the Moravia Gate, a large pass between the Carpathians and the Sudetes known as one of the major migration routes for xeric species in central Europe (Mazur 2001).

Possible hybridization

The only population of central Europe in which were present specimens morphologically identified as *C. flavipes* was located in the Carpathians (Pieniny

Mountains), well-known local refugium with the presence of xeric habitats. Interestingly, the only three examined specimens of this population expressed mixed genotypes. Despite a *C. flavipes* phenotype, only one specimen harboured a *C. flavipes* – related mtDNA, whereas the other two had *C. bameuli* – related mtDNA. Moreover, two of these specimens showed intermediate variants of their nuclear DNA (*ef1-a* gene). These findings strongly suggest that this isolated population of *C. flavipes* had a substantial gene flow with *C. bameuli* of central Europe. It has been hypothesized that when one of the two closely related species becomes rare, it could mate and hybridize with its more abundant relative (Montagna et al. 2016a). Moreover, it has been postulated that hybridization is not a rare phenomenon among *Cryptocephalus* beetles (e.g. the *Cryptocephalus sericeus* complex; Gómez-Zurita et al. 2012). The alternative scenario, which is in our opinion much less plausible, is that specimens from the Carpathians harbour ancestral polymorphisms and are suffering from incomplete lineage sorting. Further studies using larger samples, including presumable hybrid populations and using next-generation sequencing technologies, are necessary to be able to disentangle among these scenarios.

Host plants

Until now, *C. flavipes* has been considered a polyphagous species since specimens were observed and collected on different plant species, mainly deciduous trees and bushes (e.g. as *Corylus*, *Crataegus*, *Quercus*, *Crataegus*, *Salix*; Müller 1949–1953; Burlini 1955; Fogato & Leonardi 1980; Burakowski et al. 1990); however, no conclusive evidence have been provided. With regard to the recently described species *C. bameuli*, the host plants were unknown and the existence of possible variation in feeding preferences, with respect to the different geographic areas, has never been investigated.

Metabarcoding analysis on the gut content of *C. bameuli* and *C. flavipes* confirmed their polyphagy; the most commonly exploited host plants belong to the families of Betulaceae, Fabaceae and Rosaceae. The same analysis showed that the three groups of *C. bameuli* share a core diet, but some differences were observed when populations from different regions were compared, especially between the Alpine and central-eastern European populations. Such slight regional differences in the diet of polyphagous beetles have also been reported for the weevil *Centricnemus leucogrammus* Germ. (Kajtoch 2014), and it may reflect some local

feeding adaptations to different sets of plants, even if the core diet of the species is maintained. Considering the overall populations of both species, significant differences in diet composition were observed. It is noteworthy that the Alpine populations of *C. bameuli* and *C. flavipes*, inhabiting the same habitats and geographical area, share some host plants but also possess a higher number of unique hosts. Further field or lab-based observations could clarify the exact feeding preferences of these beetles.

Conclusions

The presented results provide evidence that these two sibling and sympatric taxa have experienced different histories along their evolutionary path, which may reflect their different adaptations to climatic and environmental conditions. Moreover, both species have differentiated their ecological niche (e.g. living in different xeric habitats at different elevations) and, possibly, have undergone ecological speciation through switches in their host plants. Further studies, including denser sampling as well as the use of different types of genetic markers, could help in estimating the magnitude of hybridization (frequency and geographic span) and in identifying the biological and ecological adaptations that have differentiated these sibling beetles.

Acknowledgements

We are grateful to Wiesław Babik and Katarzyna Dudek for their assistance in host plant study.

This research was funded by the grant DEC-2011/01/B/NZ8/01491 (Kajtoch Ł., National Science Centre, Poland, part concerning host plants) and by MIUR Fondo per il finanziamento delle attività base di ricerca FFABR 2017 assigned to Montagna M. We acknowledge the World Climate Research Programme's Working Group on Coupled Modelling, which is responsible for CMIP, and we thank the climate modelling group CCSM4 – University of Miami – RSMAS for producing and making available their model output. For CMIP the US. Department of Energy's Program for Climate Model Diagnosis and Intercomparison provides coordinating support and led the development of software infrastructure in partnership with the Global Organization for Earth System Science Portals.

Funding

This work was supported by the National Science Centre, Poland [DEC-2011/01/B/NZ8/01491]; MIUR Fondo per il finanziamento delle attività base di ricerca [FFABR 2017].

Disclosure statement

Authors declare no conflict of interests.

Supplementary material

Supplemental data for this article can be accessed [here](#).

ORCID

D. Kubisz  <http://orcid.org/0000-0001-8111-922X>
 G. Magoga  <http://orcid.org/0000-0003-4016-2912>
 M. A. Mazur  <http://orcid.org/0000-0001-5186-814X>
 M. Montagna  <http://orcid.org/0000-0003-4465-7146>
 R. Ścibior  <http://orcid.org/0000-0002-3556-4338>
 P. Tykarski  <http://orcid.org/0000-0003-3259-8273>
 Ł. Kajtoch  <http://orcid.org/0000-0001-7345-9400>

References

- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. *Journal of Molecular Biology* 215:403–410. DOI:10.1016/S0022-2836(05)80360-2.
- Bandelt HJ, Forster P, Röhl A. 1999. Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* 16:37–48. DOI:10.1093/oxfordjournals.molbev.a026036.
- Brunetti M, Magoga G, Iannella M, Biondi M, Montagna M. 2019. Phylogeography and species distribution modelling of *Cryptocephalus barii* (Coleoptera: Chrysomelidae): Is this alpine endemic species close to extinction? *Zookeys* 856:3–25.
- Burakowski B, Mroczkowski M, Stefańska J. 1990. *Chrzęszcz Coleoptera. Stonkowate – Chrysomelidae*, part 1. Katalog Fauny Polski, part 23, Vol. 16. Warsaw: Polish Scientific Publisher.
- Burlini M. 1955. Revisione dei *Cryptocephalus* italiani e della maggior parte delle specie di Europa (Col. Chrysomelidae). *Memorie della Società Entomologica Italiana* 34:5–287.
- Clarke K. 1993. Non-parametric multivariate analysis of changes in community structure. *Austral Ecology* 18:117–143. DOI:10.1111/j.1442-9993.1993.tb00438.x.
- De la Cadena G, Papadopoulou A, Maes JM, Gómez-Zurita J. 2017. Evaluation of bias on the assessment of diet breadth of herbivorous insects using molecular methods. *Insect Science* 24(2):194–209. DOI:10.1111/1744-7917.12303.
- Dixon P. 2003. VEGAN, a package of R functions for community ecology. *Journal of Vegetation Science* 6:927–930. DOI:10.1111/j.1654-1103.2003.tb02228.x.
- Duhaldeborde F. 1999. Description de *Cryptocephalus* (s. str.) *bameuli* n. sp., nouvelle espèce paléarctique à large répartition géographique (Coleoptera, Chrysomelidae). *Nouvelle Revue d'Entomologie (N. S.)* 16:123–135.
- Duhaldeborde F. 2018. A new *Cryptocephalus* from Turkey of the *C. flavipes* Fabricius, 1781, species-group (Coleoptera, Chrysomelidae, Cryptocephalini). *Bulletin de la Société entomologique de France* 123:259–268. DOI:10.32475/bsef_2004.
- Ehlers J, Gibbard PL. 2004. Quaternary Glaciations – Extent and Chronology, Part I: Europe. In: Ehlers J, Gibbard PL, Hughes PD. (eds) *Developments in quaternary science*, Vol. 2a. Amsterdam: Elsevier. 488 pp.
- Excoffier L, Lischer HEL. 2010. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* 10(3):564–567. DOI:10.1111/j.1755-0998.2010.02847.x.
- Fogato V, Leonardi C. 1980. Coleotteri Crisomelidi della Brughiera di Rovasenda (Piemonte). In: La Greca M. (ed.) *Quaderni sulla "Struttura delle Zoocenosi terrestri"*, Vol. 1. Roma: Consiglio Nazionale delle Ricerche. pp. 25–77. La Brughiera pedemontana.
- Gómez-Zurita J, Sassi D, Cardoso A, Balke M. 2012. Evolution of *Cryptocephalus* leaf beetles related to *C. sericeus* (Coleoptera: Chrysomelidae) and the role of hybridization in generating species mtDNA paraphyly. *Zoologica Scripta* 41(1):47–67. DOI:10.1111/j.1463-6409.2011.00500.x.
- Heise W, Babik W, Kubisz D, Kajtoch Ł. 2015. A three-marker DNA barcoding approach for ecological studies of xerothermic plants and herbivorous insects from central Europe. *Botanical Journal of the Linnean Society* 177(4):576–592. DOI:10.1111/boj.12261.
- Hijmans RJ, Cameron SE, Parra JL, Jones PG, Jarvis A. 2005. Very high resolution interpolated climate surfaces for global land areas. *International Journal of Climatology* 25(15):1965–1978. DOI:10.1002/joc.1276.
- Kajtoch Ł. 2014. A DNA metabarcoding study of a polyphagous beetle dietary diversity: The utility of barcodes and sequencing techniques. *Folia Biologica (Kraków)* 62(3):223–234. DOI:10.3409/fb62_3.223.
- Kajtoch Ł, Cieślak E, Varga Z, Paul W, Mazur MA, Sramkó G, Kubisz D. 2016. Phylogeographic patterns of steppe species in Eastern Central Europe: A review and the implications for conservation. *Biodiversity and Conservation* 25(12):2309–2339. DOI:10.1007/s10531-016-1065-2.
- Kajtoch Ł, Kubisz D, Heise W, Mazur MA, Babik W. 2015. Plant-herbivorous beetle networks: Molecular characterization of trophic ecology within a threatened steppic environment. *Molecular Ecology* 24(15):4023–4038. DOI:10.1111/mec.13278.
- Librado P, Rozas J. 2009. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25(11):1451–1452. DOI:10.1093/bioinformatics/btp187.
- Löbl I, Smetana A. 2010. *Catalogue of Palaearctic Coleoptera*, Vol. 6. Chrysomeloidea. Stenstrup: Apollo Books. 924 pp.
- Lopatin IK, Nesterova OL. 2002. A review of the *Cryptocephalus flavipes* F. species-group (Coleoptera, Chrysomelidae). *Euroasian Entomological Journal* 1:215–217.
- Mazur M. 2001. Ryjkwce kserotermiczne Polski (Curculionioidea: Nemonychidae, Attelabidae, Apionidae, Curculionidae). *Studium Zoogeograficzne. Monografie Fauny Polski* 22:1–382.
- Merow C, Smith MJ, Silander Jr JA. 2013. A practical guide to MaxEnt for modeling species' distributions: What it does, and why inputs and settings matter. *Ecography* 36(10):1058–1069. DOI:10.1111/j.1600-0587.2013.07872.x.
- Montagna M, Chouaia B, Mazza G, Prosdociemi EM, Crotti E, Merighetti V, Vacchini V, Giorgi A, De Biase A, Longo S, Cervo R, Lozzia GC, Alma A, Bandi C, Daffonchio D. 2015a. Effects of the diet on the microbiota of the red palm weevil

- (Coleoptera: Dryophthoridae). *PLoS One* 10(1):e0117439. DOI:10.1371/journal.pone.0117439.
- Montagna M, Gómez-Zurita J, Giorgi A, Epis S, Lozzia G, Bandi C. 2015b. Metamicrobiomics in herbivore beetles of the genus *Cryptocephalus* (Chrysomelidae): Toward the understanding of ecological determinants in insect symbiosis. *Insect Science* 22(3):340–352. DOI:10.1111/1744-7917.12143.
- Montagna M, Kubisz D, Mazur MA, Magoga G, Ścibior R, Kajtoch Ł. 2016a. Exploring species-level taxonomy in the *Cryptocephalus flavipes* species complex (Coleoptera: Chrysomelidae). *Zoological Journal of Linnean Society* 179:92–109.
- Montagna M, Mereghetti V, Gargari G, Guglielmetti S, Faoro F, Lozzia G, Locatelli D, Limonta L. 2016b. Evidence of a bacterial core in the stored products pest *Plodia interpunctella*: The influence of different diets. *Environmental Microbiology* 18(12):4961–4973. DOI:10.1111/1462-2920.13450.
- Montagna M, Sassi D, Giorgi A. 2013. *Pachybrachis holerorum* (Coleoptera: Chrysomelidae: Cryptocephalinae), a new species from the Apennines, Italy, identified by integration of morphological and molecular data. *Zootaxa* 3741(2):243–253. DOI:10.11646/zootaxa.3741.2.3.
- Müller G. 1949-1953. I coleotteri della Venezia Giulia: Catalogo ragionato con tabelle dicotomiche per la classificazione delle specie della Regione Adriatica Orientale, del Veneto e della Pianura Padana. II Coleoptera Phytophaga (Cerambycidae, Chrysomelidae, Bruchidae), Vol. 4. Trieste: Centro Sperimentale Agrario e Forestale. pp. 1–685.
- Papadopoulou A, Anastasiou I, Vogler AP. 2010. Revisiting the insect mitochondrial molecular clock: The mid-Aegean trench calibration. *Molecular Biology and Evolution* 27(7):1659–1672. DOI:10.1093/molbev/msq051.
- Phillips SJ, Anderson RP, Schapire RE. 2006. Maximum entropy modeling of species geographic distributions. *Ecological Modelling* 190(3–4):231–259. DOI:10.1016/j.ecolmodel.2005.03.026.
- Phillips SJ, Dudík M. 2008. Modeling of species distributions with Maxent: New extensions and a comprehensive evaluation. *Ecography* 31(2):161–175. DOI:10.1111/j.0906-7590.2008.5203.x.
- Piper R. 2002. Conservation biology of *Cryptocephalus* species and other threatened UK beetles. A thesis admitted in accordance with the requirements for the degree of Doctor of Philosophy University of Leeds School of Biology. p. 226.
- Piper RW, Compton SG. 2003. Subpopulations of *Cryptocephalus* beetles (Coleoptera: Chrysomelidae): Geographically close but genetically far. *Diversity and Distributions* 9(1):29–42. DOI:10.1046/j.1472-4642.2003.00163.x.
- Piper RW, Compton SG. 2010. Population size and dispersal ability of *Cryptocephalus nitidulus* (Linnaeus, 1758) (Col.: Chrysomelidae). *The Entomologist's Record and Journal of Variation* 122:257–264.
- Piper RW, Compton SG. 2013. How limited is dispersal in the rare beetle, *Cryptocephalus decemmaculatus* (Chrysomelidae, Cryptocephalinae)? *Journal of Insect Conservation* 17(6):1229–1235. DOI:10.1007/s10841-013-9604-6.
- Rogers A, Harpending H. 1992. Population growth makes waves in the distribution of pairwise genetic differences. *Molecular Biology and Evolution* 9(3):552–569. DOI:10.1093/oxford-journals.molbev.a040727.
- Schmitt T, Varga Z. 2012. Extra-Mediterranean refugia: The rule and not the exception? *Frontiers in Zoology* 9(1):1–12. DOI:10.1186/1742-9994-9-22.
- Stewart JR, Lister AM. 2001. Cryptic northern refugia and the origins of the modern biota. *Trends in Ecology & Evolution* 16(11):608–613. DOI:10.1016/S0169-5347(01)02338-2.
- Taberlet P, Gielly L, Pautou G, Bouvet J. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* 17(5):1105–1109. DOI:10.1007/BF00037152.
- Wang L, Xu Y. 2003. Haplotype inference by maximum parsimony. *Bioinformatics* 19(14):1773–1780. DOI:10.1093/bioinformatics/btg239.
- Warchałowski A. 2010. The Palaearctic Chrysomelidae. In: Identification keys. Warsaw: Natura Optima Dux Foundation. 1212 pp.